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Biochemical diversity of betaines in earthworms

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ABSTRACT

The ability to accumulate osmoprotectant compounds, such as betaines, is an important evolutionary feature in many organisms. This is particularly the case for organisms that live in variable environments, which may have fluctuations in moisture and salinity levels. There is, surprisingly, very little known about betaines in soil invertebrates in general, and there is almost no information about earthworms – a group that are important 'ecosystem engineers' and key indicators of soil health. Here, we describe a fast and reliable $^{1}H–^{13}C$ heteronuclear single quantum coherence (HSQC) 2D NMR approach for the metabolic profiling of a series of betaines and related metabolites in tissue extracts, and list ^{1}H and ^{13}C chemical shifts for the trimethylammonium signal for 23 such compounds. The analysis of ten different species from three different families (Lumbricidae, Megascolecidae and Glossoscolecidae) showed an unexpected diversity of betaines present in earthworms. In total ten betaines were identified, including hydroxyproline-betaine, proline-betaine, taurine-betaine, GABA-betaine and histidine-betaine, and a further eleven as-yet unassigned putative betaine metabolites detected. The findings clearly indicate a hitherto-unappreciated important role for betaine metabolism in earthworms.

1. Introduction

Betaines – trimethylammonium derivatives of amino acids and related compounds – are an important set of metabolites for many organisms, including microbes, algae, plants, and animals, including humans [1–4]. They play a pivotal role in osmoprotection of cells and tissues to maintain cellular homeostasis, and to protect them against environmental stresses like high salinity and extreme temperatures. Additionally, they serve as a catabolic resource of methyl groups in different biochemical pathways (transmethylation).

Betaines are well studied in bacteria [5], plants and algae [1,6], but less is known about their occurrence in many other species. This is true for invertebrates, and in particular terrestrial invertebrates. Earthworms are a classic example where fundamental knowledge about their occurrence and distribution is lacking. Earthworms can survive and flourish in soils with both high and low extremes of water stress [7]. Soil moisture is considered to be the primary factor limiting survival of different earthworm species [8,9], therefore they must preserve an efficient osmoregulatory system. Surprisingly little is known about betaines and other trimethylammonium compounds in earthworms. Our knowledge of which compounds may be present and their natural distribution is far from complete, but for instance metabolic profiling approaches by ^{1}H NMR have detected both glycine-betaine and choline in *Lumbricus rubellus* [10] and *Eisenia fetida* [11]. This lack of knowledge motivated us to study the landscape of betaine compounds in earthworms. Additionally, we aimed to develop a fast and reliable method for the detection of these compounds, which can be easily applied to other sample types.

Betaines are zwitterionic quaternary ammonium compounds and so are not trivial to analyze by common separation methods such as reversed phase HPLC, although methods have been developed using hydrophilic interaction liquid chromatography [12] or a pentafluorophenylpropyl stationary phase [13] to separate betaines. More time-consuming protocols, including previous derivatization of betaines, can also be applied to improve HPLC retention characteristics [14]. In contrast, nuclear magnetic resonance (NMR) spectroscopy can also be used for analysis of betaines, and does not require physical separation of metabolites [1]. Betaine metabolites give rise to singlet resonances from the trimethylammonium group; because these are based on nine protons, and there is no resonance splitting, these compounds have relatively low detection levels by ^{1}H NMR. However, it is difficult to assign betaines in 1D spectra based on the trimethylammonium peak alone; unfortunately, these are often the only peaks easily detected in crude cell/tissue extracts (especially for low-concentration compounds) because the other compound resonances are obscured by overlapping signals from other metabolites. Either homonuclear or heteronuclear two-dimensional NMR experiments are required for confident assignment of betaine metabolites in a given sample; in particular, heteronuclear $^{1}H–^{13}C$ methods make advantage of the inherently wide chemical shift distribution of the ^{13}C nucleus.

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However, these can be time consuming, especially if aiming to give high-resolution spectra across the full range of proton and carbon shifts found in tissue extracts. Fortunately, as already described, betaines possess a characteristic trimethylammonium resonance, and spectral acquisition times can be drastically reduced (while maintaining resolution in both dimensions) by concentrating on this spectral region.

We present here an analytical approach that provides both qualitative and quantitative data, by combining short 1D and 2D HSQC NMR acquisitions of spectra from tissue extracts. We also provide a reference database for a range of amino acid derived betaines to consistently identify as many as possible compounds in various samples, and apply this to characterize the betaine profiles in 10 different earthworm species.

2. Materials and methods

2.1. Earthworm species

Lumbricus rubellus (Lumbricidae) worms were a gift from David Spurgeon (CEH Wallingford, UK). *Aporrectodea chlorotica* (Lumbricidae) worms were collected from field populations within the UK. *Lumbricus terrestris*, *E. fetida*, and *Dendrobaena veneta* (all Lumbricidae), were purchased from Blades Biological Ltd. (Edenbridge, UK). Four additional species from two families were collected in glasshouses with tropical climate and flora based in the Royal Botanic Gardens, Kew, London (UK). These were *Amynthas rodericensis, Amynthas corticis* (putative assignment – identification was to a lower level of confidence than for the other species), *Pithemera bicincta* (all Megascolecidae), and *Pontoscolex corethrurus* (Glossoscolecidae). *Amynthas gracilis* (Megascolecidae) worms were a gift from Peter Kille (University of Cardiff, UK) and originally collected from the island of Furnas (Portugal).

2.2. Metabolite extraction

The worms were frozen in liquid N_2 and the frozen tissue cryogenically ground using a FreezerMill 6870 (Spex SamplePrep, Stanmore, UK). The milled tissue was extracted with a method described in detail elsewhere [15]. Briefly, 20 mL ice cold acetonitrile:methanol:water 2:2:1 (vol./vol.) were added to 1–3 g tissue powder and mixed in a vortexer for 1 min, freeze–thawed, and mixed again and centrifuged (4000*g*, 5 min). The supernatant was transferred into glass tubes and dried down in a rotary vacuum concentrator (Eppendorf, Cambridge, UK).

2.3. NMR analysis

The dried extracts were resuspended in 650 μL D_2O with 0.1 M phosphate buffer, pH 7.0, containing DSS as internal standard (5 mM). Samples were centrifuged at 10,000 rpm for 2 min and the supernatant was transferred into 5 mm NMR glass tubes. Samples were analyzed with a 800 MHz Bruker Avance spectrometer equipped with a triple resonance cryoprobe. The sample temperature was set to 300 K; the temperature was not calibrated directly for this experiment, but is calibrated for this probe at regular intervals. For all samples a one-dimensional ^{1}H NMR spectrum with solvent suppression on the residual water peak was acquired

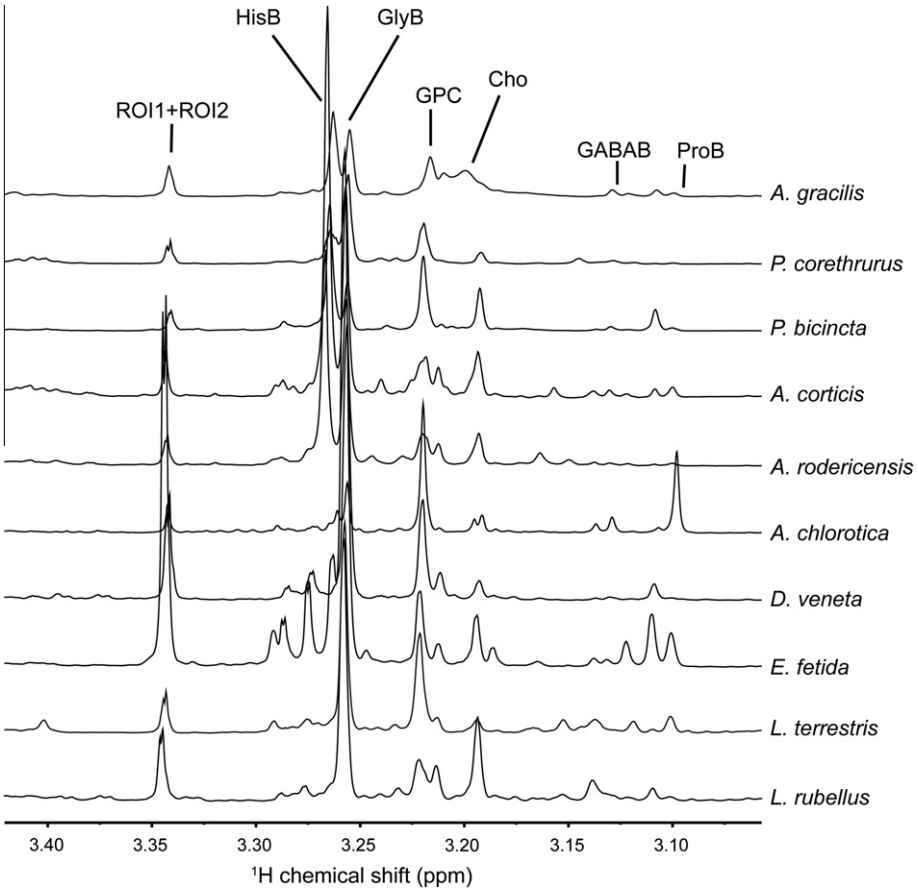

Fig. 1. One-dimensional ^{1}H NMR spectra of all earthworm species, showing a region for potential betaine signals. Selected signals are indicated, proline-betaine (ProB), GABA-betaine (GABAB), choline (Cho), glycerophosphocholine (GPC), glycine-betaine (GlyB), histidine-betaine (HisB), unidentified metabolites ROI1 and ROI2.